Oral treatment of unconjugated hyperbilirubinemia

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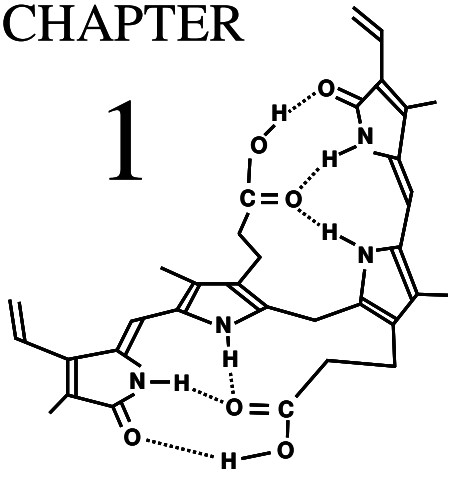
General introduction

CHAPTER 1



1. BILIRUBIN

The term *bilirubin* is derived from the Latin words for bile (*bilis*), and red (*ruber*). Städeler[1] first used it in 1864 to describe the orange-red colored bile pigment. When bilirubin accumulates in the body it causes a yellow discoloration of the skin, sclerae and other tissues, referred to as *jaundice* (from the French *jaunisse*) or *icterus* (from the Greek *ikteros*), and high levels of bilirubin in the blood, hyperbilirubinemia. This thesis focuses on oral treatment options for unconjugated hyperbilirubinemia. This general introduction successively describes bilirubin metabolism, unconjugated hyperbilirubinemia, Crigler-Najjar disease, kernicterus, current treatment options, the Gunn rat animal model, and two of our proposed treatment options: orlistat, and bile salts. The outline of this thesis is presented in chapter 2.

2. BILIRUBIN METABOLISM

2.1. Bilirubin production

Bilirubin is the end product of heme catabolism. The major source of heme (75-80%) is hemoglobin, from breakdown of erythrocytes. Other heme sources include cytochromes, peroxidase, catalase, myoglobin, and ineffective erythropoiesis.[2;3] The life span of erythrocytes is approximately 120 days in adults, 90 days in neonates and 50-60 days in rats.[4] Senescent erythrocytes are removed from the circulation and destroyed in the reticuloendothelial system (RES), mainly localized in the spleen, liver and bone marrow. In the RES, heme is phagocytized by macrophages. Macrophages contain microsomal heme oxygenase and cytosolic biliverdin reductase, two essential enzymes for degradation of heme to bilirubin. Heme oxygenase catalyzes the first step in heme degradation: the opening of the porphyrin ring structure at the α-methene bridge (Figure 1).

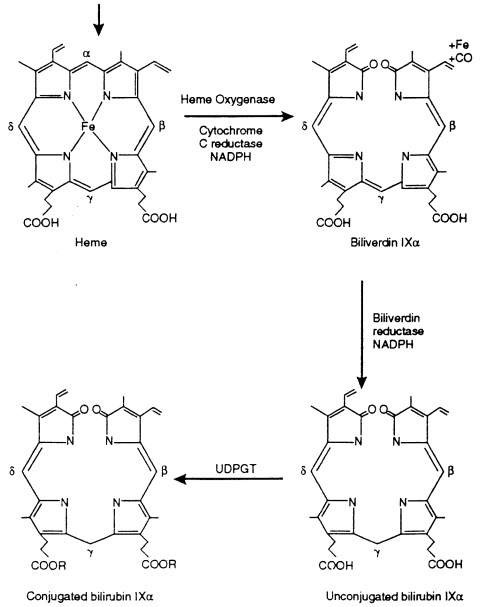


Figure 1. Bilirubin production from heme catabolism.

The intermediate blue-green pigment formed, biliverdin IXα, is water-soluble and nontoxic. The iron (Fe) is recycled and carbon monoxide (CO) is excreted by the lungs.[5] In mammals, biliverdin IXα is reduced by NADPH-dependent biliverdin reductase to produce bilirubin IXα, also known as unconjugated bilirubin **(UCB)**. Why the nontoxic, water-soluble biliverdin is converted to the non-water-soluble and potentially toxic UCB is unclear. One hypothesis involves the need for products of fetal heme degradation to cross the placenta.[6;7] Biliverdin cannot, whereas the more lipophilic UCB can cross the placenta. Another reason could be the antioxidant properties of UCB[8] (see paragraph 3.3).

UCB production can be assessed by measurement of CO formation. Conversion by heme oxygenase of one heme molecule to biliverdin produces one molecule of CO. Production rate of UCB is approximately 6-8 mg/kg per 24 hours in healthy full-term infants, and 3-4 mg/kg per 24 hours in healthy adults.[9;10] Infants produce more UCB per kg body weight because of their higher red blood cell (RBC) count, the relatively larger fraction of hepatic heme proteins, and the shorter life span of fetal RBC's. Fetal hemoglobin (HbF), which has a higher affinity for oxygen than "adult" HbA, is broken down postnatal in the relatively oxygen-rich environment. Apart from CO measurements, UCB production rate can be derived from turnover of radioisotopically labeled bilirubin, under steady-state conditions.

2.2. Bilirubin chemistry

The systemic name of UCB (bilirubin IXα) is 1'8'-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-*a*,*c*-dipropionic acid (4,5).[11;12] Its molecular weight is 584,7 gram. UCB is a nearly symmetrical tetrapyrrole, consisting of two rigid, planar dipyrroles joined by a methylene (-CH_2-) bridge at carbon atom 10 (Figure 2).[13]

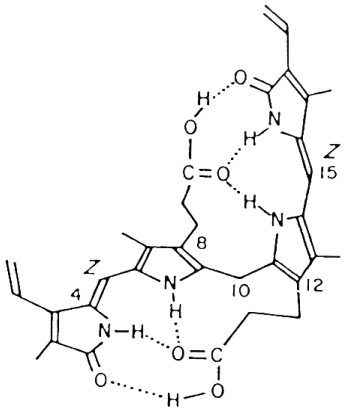


Figure 2. Structure of unconjugated bilirubin. From McDonagh and Lightner.[14]

UCB preferably has a "ridge-tile" conformation, *i.e.* is shaped like a partially open book. UCB structure was identified by analysis of X-ray diffraction.[15] Six internal hydrogen bonds make the molecule insoluble in water because the hydrophilic polar COOH and NH groups

are not available for attachment of H_20 and the hydrophobic hydrocarbon groups are on the outside of the molecule.[16] When the hydrogen bonds are opened at, for example, an alkaline pH or by addition of (m)ethanol, diphyllin or caffeine, UCB becomes more labile, more polar and water-soluble. This allows UCB to react rapidly with the diazo reagent, the basis for measurement of unconjugated or indirect bilirubin by the Van den Bergh reaction.[17] UCB exists as three species with different degrees of ionization (H_2B or diacid, HB^- or monoanion, and B^{2-} or dianion,[13] see paragraph 3.1 for details). The integrity of the hydrogen-bonded structure requires the interpyrrolic bridges at positions C4 and C15 to be in the *trans* or *Z* configuration (*Z* for *zusammen*). During phototherapy this configuration is disrupted (see paragraph 7.1).

2.3. Bilirubin transport

Once the hydrophobic UCB leaves the reticuloendothelial system, over 99.9% is bound in plasma to albumin in a non-covalent fashion and transported to the liver. Albumin has a high affinity binding site for UCB. Beyond a molar ratio of 1:1, which is equivalent to a plasma UCB concentration of approximately 600 µmol/l, UCB can bind to albumin at additional lower affinity binding sites.[18;19] In the absence of albumin, the aqueous solubility of UCB at pH 7.4 is less than 0.1 µmol/l, emphasizing the importance of albumin for preventing unbound (*i.e.* free) UCB, which is considered toxic (see paragraph 3). Recently, high-density lipoprotein (HDL) has been reported to be the principal nonalbumin carrier of UCB in human plasma. The affinity of HDL for UCB is primarily the result of binding to apolipoprotein D.[20]

2.4. Hepatic uptake of bilirubin

Albumin delivers UCB to the liver where fenestrae in the sinusoidal endothelial cells allow albumin-bound substances to reach the subendothelial space of Disse.[21] Hepatocytes have a highly efficient capacity for removing UCB from plasma. The uptake of UCB into the hepatocyte results from dissociation from albumin and transfer across the plasma membrane.[16] This transfer of UCB is carrier mediated, although controversy exists regarding the exact mechanism.[21;22] Several proteins have been suggested as putative UCB transporter, including the organic anion transport protein (Oatp2 / Slc21a6)[23;24] and the bilirubin/BSP binding protein (BBBP) which also transports other organic anions such as bromosulfophthalein (BSP).[25] The role of Oatp2 is disputed; several other Oatp's have been implicated. Bilitranslocase (BTL)[26] has also been implicated but evidence for its existence and structure is questionable.

Once within the hepatocyte, UCB is bound by the major cytosolic binding protein for UCB, glutathione S-transferase, traditionally referred to as ligandin or Y-protein.[27;28] UCB flux across the hepatocyte membrane is bidirectional. Binding to glutathione S-transferase decreases the unbound fraction and thereby the reflux of UCB and conjugated bilirubin back into plasma.[29;30]

2.5. Bilirubin conjugation

Figure 3 shows metabolism of UCB in the hepatocyte. In order to excrete bilirubin efficiently into bile, conjugation is required to convert the non-polar, water-insoluble UCB (at pH 7.4) to a water-soluble conjugate. Glucuronic acid is the major conjugating group.[31] Traces of other conjugates (e.g. glucose and xylose conjugates) have been identified in human bile,[32] and higher proportions of glucose and xylose conjugates are present in rat and dog bile. Bilirubin glucuronides are present as mono- and diglucuronides. The enzyme bilirubin-uridine diphosphoglucuronosyltransferase (UDPGT, UGT1A1, EC 2.4.1.17), primarily located in the endoplasmic reticulum, catalyzes the transfer of one or two glucuronic acid(s) from UDP-glucuronate (UDPGA) to UCB, forming, respectively, bilirubin monoglucuronides (BMG, ~20%) or bilirubin diglucuronides (BDG, ~80%) that are excreted into bile.[33] Absence of UGT1A1 in Crigler-Najjar disease results in unconjugated hyperbilirubinemia (see paragraphs 4 and 5).

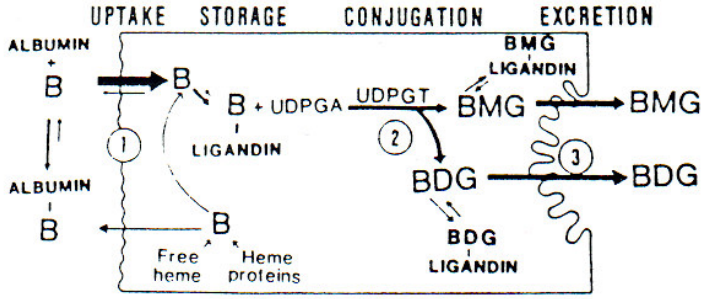


Figure 3. Hepatic metabolism of UCB. (1). UCB (B) is transferred from plasma into the hepatocyte and bound to ligandin. (2). UDP-glucuronosyltransferase (UDPGT, UGT1A1) catalyses the transfer of glucuronic acid from UDP-glucuronate (UDPGA) to bilirubin, forming bilirubin monoglucuronide (BMG) and diglucuronide (BDG) that are excreted into bile, (3) across the canalicular membrane. Ligandin is gluthathion-S-transferase (see paragraph 2.4). From Roy Chowdhury et al.[11]

2.6. Bilirubin excretion

Conjugation is an important step in UCB catabolism. Efficient biliary secretion of bilirubin requires conversion to polar conjugates. A very small amount of UCB is excreted into bile without conjugation, where it rapidly associates with mixed micelles.[11;34] UCB in bile is seldom more than 2% of total bilirubin and is believed to derive in large part from hydrolysis of secreted conjugates in the biliary tree. Conjugated bilirubin leaves the hepatocyte via Mrp2 (multidrug resistant protein 2, Abcc2). Mrp2 is an ATP-dependent transporter that carries conjugated bilirubin across the canalicular membrane into the biliary tree. Absence of Mrp2 in patients with Dubin-Johnson syndrome, and in analogous rat models (the TR- rat and the Eisai hyperbilirubinuria rat), causes conjugated hyperbilirubinemia.[35-37] However, Mrp2 cannot be the only canalicular transporter that is able to excrete conjugated bilirubin, because in the TR- rat organic anion transport was found to be preserved.[38] Mrp3 (multidrug resistant protein 3, Abcc3) is considered an important candidate for basolateral excretion of conjugated

bilirubin.[39] Conjugated bilirubin is retained in hepatocellular and cholestatic disorders. Increased plasma levels of conjugated bilirubin result in formation of a bilirubin-albumin complex called δ-bilirubin,[40] which reacts directly with diazo reagents, as does conjugated (*i.e.* direct) bilirubin.[17]

Multidrug resistant protein 1 (Mrp1, Abcc1) is a proven exporter of UCB that requires glutathione as a co-factor. Mrp1 protects cells against UCB-induced cytotoxicity.[41-44]

2.7. Intestinal metabolism and enterohepatic circulation of bilirubin

Conjugated bilirubin is hydrolyzed in the intestine to UCB, which can be reabsorbed into the enterohepatic circulation (EHC,[45;46] Figure 4). Hydrolysis of conjugated bilirubin to UCB can occur nonenzymatically under the influence of mild alkaline conditions as in the duodenum or jejunum,[47] and enzymatically by β-glucuronidase. Endogenous tissue β-glucuronidase exists in the enteric mucosa and liver,[48;49] but the major part of enzyme activity is of bacterial origin.[50;51] In neonates, a relative lack of bacterial flora and a high mucosal β-glucuronidase activity increase the enterohepatic circulation of UCB. β-glucuronidase is present in human breast milk and was thought to exaggerate jaundice in breastfed infants.[52] However, the small amounts of enzyme in milk relative to the large amounts of mucosal β-glucuronidase would not be expected to add much to the overall activity.[16] UCB in the intestine not only results from deconjugation of conjugated bilirubin. UCB can also diffuse from the blood into the intestinal lumen across the mucosa,[53;54] particularly when plasma UCB levels are high (*e.g.* neonatal jaundice; Crigler-Najjar disease, see paragraph 5). Preventing enterohepatic circulation of UCB is one of the strategies for treatment of unconjugated hyperbilirubinemia (see paragraph 7.5).

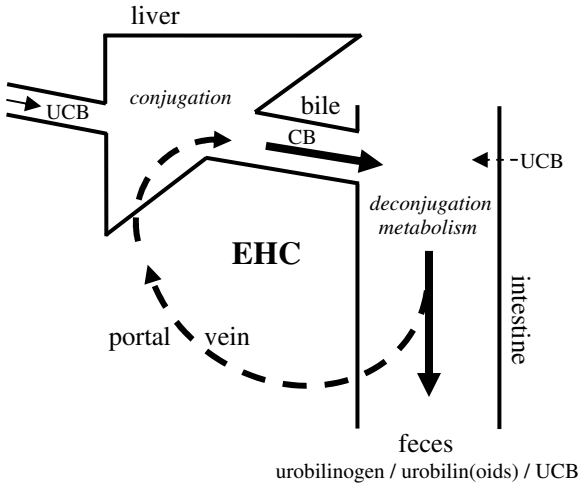


Figure 4. Enterohepatic circulation (EHC) of bilirubin. UCB is conjugated in the liver. Conjugated bilirubin (CB) is excreted into bile. CB is deconjugated to UCB via β-glucuronidase, and partly metabolized in the intestine to urobilinogen and urobilin(oids), which are excreted in the feces. Part of the UCB is reabsorbed into the EHC. Particularly under conditions of severe unconjugated hyperbilirubinemia (*e.g.* Crigler-Najjar disease), UCB can diffuse from the blood into the intestinal lumen across the mucosa (see Figure 1 in Chapter 2).

Conjugated bilirubin must be hydrolyzed to UCB before the tetrapyrrole ring can be reduced to the colorless urobilinogens by intestinal anaerobic bacteria (3 *Clostridia* species and *Bacteroides fragilis*).[55;56] Urobilinogen can be oxidized to the yellow-orange urobilin. The brown color of feces is due to dipyrrolic oxidative derivates of UCB, the mesobilifuscins. Absence of urobilinogen in feces and urine indicates complete obstruction of the bile duct. Oxidation-reduction of the various unsaturated bonds in bilirubin results in a large family of related colorless reduction-oxidation products known as urobilinoids.[57] The formation of urobilinoids is important for the removal of bilirubin from the body because the majority of urobilinoids is excreted via the feces. A small portion is reabsorbed across the intestinal mucosa into the enterohepatic circulation and subsequently excreted by liver and kidney. Urobilinogen can also undergo enterohepatic circulation.[58;59] Conjugated bilirubin cannot be reabsorbed into the portal circulation.

2.8. Bilirubin oxidation

When conjugation of UCB is deficient, as in Crigler-Najjar disease (paragraph 5), or in the animal model for this disease, the Gunn rat (paragraph 8), part of the UCB can be catabolized via an alternative metabolic route: oxidation. Oxidation of UCB leads to more polar metabolites that can be excreted into bile. Hydroxylated products have been identified in bile of Gunn rats.[60;61] Microsomal cytochrome P450 enzymes such as Cyp1a1 and Cyp1a2 catalyze oxidation of UCB. In young Gunn rats, Cyp1a1 and Cyp1a2 are markedly upregulated.[62] Stimulation of P450-1a1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased plasma UCB levels in Gunn rats by approximately 60%.[63] Mitochondrial bilirubin oxidase, a constitutive non-inducible oxidase, was found in liver, intestine and kidney.[64;65] Enzymatic oxidation of bilirubin has also been reported in brain, lung, heart and skeletal muscle.[16;66]

3. BILIRUBIN TOXICITY AND ANTIOXIDANT PROPERTIES

3.1. Bilirubin neurotoxicity

It is generally accepted that UCB bound to albumin or other plasma (lipo)proteins is not toxic. Unbound, *i.e.* free UCB is toxic when the concentration is higher than its aqueous solubility (70 nM)[67] and the free UCB is bound to brain cells. However, free UCB concentrations of 40 nM (*i.e.* below aqueous solubility) also showed toxicity to cultured astrocytes.[41] The diacid (H_2B) is considered the toxic agent because the dianion (B^{2-}) and monoanion (HB^-) do not diffuse readily into cells.[19;68;69] B^{2-} and HB^- are relatively more water-soluble because of, respectively, 2 and 1 open internal hydrogen bond(s). At pH 7.4 in plasma, there is <2% dianion and >80% diacid.[13] Free H_2B can diffuse bidirectionally, passively and rapidly across membranes,[70] including the blood-brain barrier.[71] The mechanism of UCB neurotoxicity is not fully understood. Brain damage probably results from a combination of risk factors. Free

UCB not only enters brain tissue when the UCB binding capacity in plasma is exceeded, or when displacing substances (*e.g.* sulfonamides or free fatty acids) compete for bilirubin-binding sites on albumin.[16] At bilirubin/albumin ratio's below 1.0 toxicity can also occur if free UCB levels increase steeply.[72] Acidosis is considered a risk factor for the development of bilirubin encephalopathy, although the effect of acidosis on UCB-albumin interaction is controversial.[73] The adverse effects of acidosis appear secondary to rapid deposition of insoluble H_2B precipitates in tissues. An increased permeability of the blood-brain barrier via disruption of tight junctions by hyperosmolality, hypercapnia, asphyxia or hypertension can increase entry of free UCB (and even albumin-bound UCB) in the brain.[74] Possibly hyperthermia and septicemia have similar effects.[75] Recently, it has been suggested that transporter molecules in the blood-brain barrier actively pump free UCB out of the central nervous system and maintain a concentration gradient of UCB from cerebrospinal fluid to plasma.[69] Indirect support for this hypothesis can be derived from the observation that UCB induces expression and translocation of multidrug resistance-associated protein 1 (Mrp1, Abcc1) in astrocytes.[41] Intracellular UCB levels may also be diminished by oxidation, conjugation or binding to cytosolic proteins (gluthathione-S-transferases).[69] Regional UCB deposits in the brain are probably mainly explained by regional differences in exporters of UCB,[67;69] but may also relate to differences in lipid composition, blood flow[76] or bilirubin oxidation.[77] Sex-specific regional differences in brain UCB content were demonstrated in Gunn rat pups.[78] Kernicterus, deposition of UCB in brain nuclei, is described in paragraph 6.

The exact mechanism of UCB toxicity at the cellular level is still under debate. In the past, UCB was shown to impair mitochondrial function and to interfere with RNA/DNA synthesis and carbohydrate metabolism in the brain. However, these studies were performed at extremely elevated, *i.e.* not physiologically relevant, free UCB concentrations. More recent papers showed that UCB decreases cell membrane potential and disrupts transport of neurotransmitters.[67;79] UCB also inhibits protein phosphorylation in brain membranes and glycolysis in brain,[76;80] and interferes with intracellular calcium homeostasis[81] and glutamate efflux.[82] Microglia cells and astrocytes damaged by UCB produce cytokines that may contribute to brain toxicity.[83-85] Free UCB induces apoptosis at levels as low as 71-85 nmol/l.[86] Since damage to neurons and astrocytes can occur at free UCB concentrations near or modestly above aqueous saturation,[69] treatment of jaundiced neonates should be intensified if at physical examination early signs of bilirubin encephalopathy are detected, even if plasma UCB levels are only moderately elevated.

3.2. Bilirubin toxicity to other organs

Apart from the brain, UCB may also have deleterious effects on other organs. Gunn rats develop kidney damage. Bilirubin crystals and necrosis result in impaired urinary concentration with polyuria.[87;88] In patients with Crigler-Najjar disease overt renal dysfunction has not been described. Reduced kidney function has been found in jaundiced

neonates.[89] The liver is relatively resistant to UCB toxicity. This is being re-examined, but may be due to the high conjugating activity or high degree of protein binding in this organ.[80] Dental enamel dysplasia or green discoloration of the teeth may occur.[90] Patterns of bilirubin deposition have also been found in heart, lung, adrenal, pancreas, testes and skin.[91;92] UCB can inhibit cartilage metabolism and growth *in vitro*,[93] and may inhibit cellular immune responses.[94]

3.3. Bilirubin as antioxidant

Bilirubin may not only be a potentially toxic metabolite from heme degradation, it may also be good for you.[95] The first suggestion that UCB might have a physiologic function was made in 1937 when UCB appeared to be part of a protective mechanism designed to overcome (pneumococcal) infection.[96] Subsequently many have demonstrated antioxidant properties of UCB. UCB inhibits auto-oxidation of unsaturated fatty acids,[97] scavenges peroxyl radicals,[8] may prevent oxidative membrane damage, and detoxifies singlet oxygen.[95] Infants with illnesses believed to enhance free-radical production (e.g. sepsis, asphyxia) had a significantly lower daily rise in mean plasma bilirubin levels than control infants, consistent with the hypothesis that bilirubin is consumed as an antioxidant.[16;98] Conjugated bilirubin and biliverdin also have antioxidant properties.[95;99] Exogenous bilirubin had protective effects on ischemia-reperfusion injury in the isolated, perfused rat kidney,[100] and biliverdin administration protected against endotoxin-induced acute lung injury in rats and protected rat livers from ischemia and reperfusion injury.[101;102] Heme oxygenase induction protected human hepatocytes against warm and cold hypoxia. The proposed mechanisms by which heme oxygenase exerts its cytoprotective effects include its abilities to degrade the pro-oxidative heme, to produce biliverdin and subsequently bilirubin, and to generate carbon monoxide, which has antiproliferative and anti-inflammatory as well as vasodilatory properties.[103]

In vitro exposure of neurons and astrocytes to free UCB showed neuroprotection at free UCB levels below aqueous saturation (70 nM).[104] UCB was shown to inhibit oxidation of low density lipoprotein more effectively than a vitamin E analogue,[105] hence it was postulated that UCB may reduce atherogenesis. Elevated bilirubin levels,[106-111] and (inducers of) heme oxygenases[103;112] are associated with a diminished risk of atherosclerosis and appear also negatively related to the risk of cancers and demyelinating neuropathies.[113]

4. UNCONJUGATED HYPERBILIRUBINEMIA

Hyperbilirubinemia can either be unconjugated or conjugated, or involves elevation of both UCB and conjugated bilirubins, as the vast majority of conjugated hyperbilirubinemias. Conjugated hyperbilirubinemia always involves a pathophysiological mechanism located after the level of hepatic conjugation, including secretory defects and bile duct obstructions.

Examples of conjugated hyperbilirubinemia include inherited syndromes with reduced biliary secretion of conjugated bilirubin (Dubin-Johnson syndrome, Rotor syndrome), obstructive jaundice (tumor/stones), and Benign Recurrent Intrahepatic Cholestasis.[11] This introduction will be limited to unconjugated hyperbilirubinemia, which can result from increased UCB production, decreased hepatic uptake, decreased conjugation or increased enterohepatic circulation of UCB.

Unconjugated hyperbilirubinemia becomes clinically apparent with visible jaundice at plasma bilirubin levels of about 85 µmol/l.[16] Normal plasma total bilirubin levels in human adults range from 5 to 17 µmol/l.[114] Neonatal jaundice starts at the head and progresses in a cephalocaudal manner to the trunk, arms, legs, palms and soles.[16] Increased heme catabolism contributes to jaundice in the first days after birth.[115] For the majority of neonates, unconjugated hyperbilirubinemia is a benign transitional phenomenon of no overt clinical significance.[116] However, in some cases and in the presence of risk factors such as prematurity, hemolytic disease or inherited deficiency of UGT1A1, plasma UCB concentration may rise to hazardous levels leading to kernicterus or bilirubin-induced neurologic damage (BIND). These entities and treatment of unconjugated hyperbilirubinemia will be discussed in paragraphs 6 and 7. Although several guidelines for the management of unconjugated hyperbilirubinemia have been published, definitive data on “safe” plasma UCB concentrations have not been established. Controversy remains regarding the toxicity of moderately elevated plasma UCB levels,[117] and regarding pro’s and con’s of too strict versus not strict enough guidelines, both of which may increase the risk of kernicterus. The causes of unconjugated hyperbilirubinemia will now be discussed consecutively in more detail.

4.1. Increased bilirubin production

Neonates have an increased UCB production compared with adults, mainly because of a higher erythrocyte count and a shorter erythrocyte life span (see also paragraph 2.1). Other causes of increased bilirubin production include hemolysis due to blood group incompatibility, due to structural or biochemical erythrocyte defects, or due to sepsis. Extravasation of blood (cephalhematoma, intracranial hemorrhage) and polycythemia contribute to a high bilirubin load.

4.2. Decreased hepatic uptake

A reduced capacity of net hepatic uptake may contribute to the pathogenesis of physiologic jaundice. In newborn monkeys, deficiency of ligandin and reduced clearance of BSP were demonstrated in the first days of life.[118] In humans, this deficiency is of less importance than an absolute deficiency of bilirubin conjugation,[119] or a relative deficiency of conjugation due to a mismatch between increased supply of UCB in the neonatal period and conjugation capacity. In Gilbert syndrome (see paragraph 4.3) some patients have a reduced hepatic uptake of bilirubin.[11;120;121]

4.3. Decreased conjugation

In the first ten days of life, UGT1A1 activity is usually less than 0.1% of adult values.[122] Then UGT1A1 activity increases exponentially to adult values at 6 to 14 weeks of life. The postnatal increase in plasma UCB levels appears to play an important role in the initiation of bilirubin conjugation.[123] Three heritable forms of deficient UGT1A1 activity have been described. Crigler-Najjar disease type I and II will be discussed in paragraph 5. Gilbert syndrome, described in 1901 by Gilbert,[124] is a mild recurrent unconjugated hyperbilirubinemia that usually does not become manifest until after the second decade of life. In Gilbert syndrome UGT1A1 activity is approximately 20-30% of normal and in some patients an additional reduced hepatic uptake of bilirubin has been demonstrated.[11;16;120;121] The prevalence of patients with Gilbert syndrome ranges between 2 and 12%.[125;126] The mode of inheritance is most likely autosomal recessive.[127;128] A polymorphism (an extra TA in the TATAA box) in the promoter region of the UGT1A1 gene appears to be necessary for Gilbert syndrome but not sufficient for the complete manifestation of the syndrome.[128;129] To increase plasma UCB concentration, a concomitant decrease in hepatic uptake and/or increase in UCB production is needed. Some patients have an increased bilirubin turnover rate due to subclinical hemolysis.[125] The majority of patients is anicteric because plasma UCB concentrations are usually less than 50-85 μmol/l. Intercurrent illnesses and fasting may exaggerate the unconjugated hyperbilirubinemia and cause manifest jaundice. Administration of phenobarbital reduces the unconjugated hyperbilirubinemia, but does not enhance UGT1A1 activity in Gilbert patients.[11]

4.4. Increased enterohepatic circulation

Delayed intestinal transit due to starvation, delayed passage of meconium, pyloric stenosis or Hirschsprung's disease increases the enterohepatic circulation of UCB.[130] Increased intestinal motility allows less time for UCB absorption. Frequent feedings[131] and rectal stimulation[132] are associated with lower plasma UCB levels. The absence of anaerobic bacterial flora in the neonatal intestine, with limited conversion of UCB to urobilinogen, greatly enhances the enterohepatic circulation of UCB. In older children and adults a comparable situation occurs during treatment with broad-spectrum antibiotics that suppress the anaerobic flora.[56]

Breast feeding enhances the enterohepatic circulation of UCB via several mechanisms. The first few days, intake is limited, leading to delayed passage of meconium and decreased stool weight.[131;133] Breast milk contains β-glucuronidase which converts conjugated bilirubin to UCB.[52] Breast milk is thought to alter the bacterial colonization of the intestine leading to decreased formation of urobilinogen.[134;135] UGT1A1 polymorphisms or Gilbert syndrome may be an underlying cause of breast milk jaundice.[130;136] Free fatty acids in breast milk have been suggested to contribute to neonatal jaundice through inhibition of UGT1A1.[137;138] However, it is not easy to envision how intestinal free fatty acids would affect the liver, given the physiological post-absorptive transport of intestinal fatty acids in the form of chylomicron

triglycerides. Rather, the association between jaundice and free fatty acids in milk may be based on the presence of lipase activity in breast milk. Lipases in breast milk, in particular bile salt stimulated lipase, may increase the amount of free fatty acids and enhance fat absorption. According to our hypothesis that unabsorbed fat captures UCB in the intestine, a lower fraction of unabsorbed fat in the intestinal lumen will result in less UCB capture, more enterohepatic circulation and subsequently less fecal excretion of UCB.[139] This hypothesis is the basis for the research described in this thesis and is discussed in more detail in chapter 2.

5. CRIGLER-NAJJAR DISEASE

Crigler-Najjar disease type I and II are autosomal recessive inherited diseases characterized by permanent unconjugated hyperbilirubinemia since birth. Crigler-Najjar disease type I was first described in 1952 and is caused by a complete absence of UGT1A1 activity.[140] Untreated, plasma UCB levels would range between 350-800 µmol/l and patients would develop kernicterus and die (see paragraph 6 for kernicterus). Type II Crigler-Najjar disease was defined in 1962 by Arias.[141] In type II patients UGT1A1 activity is usually less than 5% of normal. Plasma UCB concentrations are generally below 350 µmol/l. The diagnosis Crigler-Najjar disease is made using high-performance liquid chromatography (HPLC) analysis of plasma and duodenal bile (Figure 5) and by evaluating the response to phenobarbital.[142] Bile of type I patients contains virtually no conjugated bilirubin, whereas bile of type II patients contains predominantly mono-conjugates and some di-conjugates.[142] Phenobarbital enhances residual enzyme activity and the two other steps in hepatic bilirubin metabolism (see paragraph 7.3). In type II patients, plasma UCB concentration decreases by approximately 30% or more, a few days after phenobarbital is started.[142;143] Type I patients show no response to phenobarbital, or a small response due to induction of ligandin and a partial shift of UCB to the liver, as seen in Gunn rats.[144]

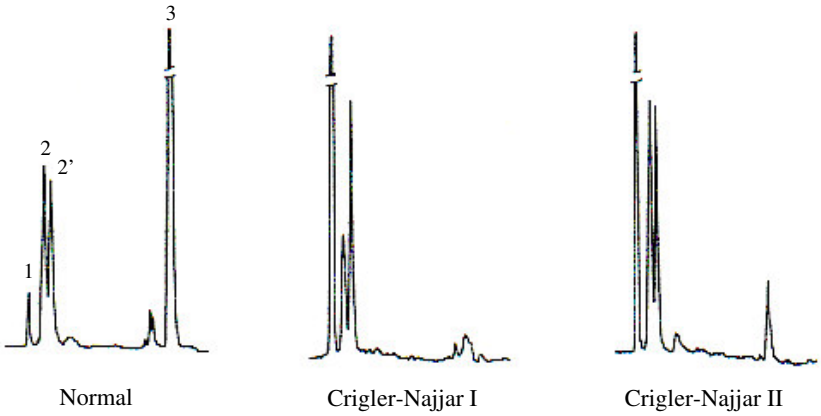


Figure 5. Bilirubin composition in bile, analyzed by HPLC. Normally, bile contains high amounts of conjugated bilirubin. In Crigler-Najjar disease type I, the bile contains virtually no conjugated bilirubin, and in type II disease predominantly mono-conjugates and some di-conjugates. Peak 1, unconjugated bilirubin; peak 2 and 2', bilirubin monoglucuronides (C8,C12 isomer); peak 3, bilirubin diglucuronide. Adapted from Sinaasappel et al.[142]

The prevalence of Crigler-Najjar disease is estimated at 1:1,000,000.[145] In the Netherlands there are approximately 20 patients. The gene encoding for UGT1A1 lies on chromosome 2. Mutations in any of 5 exons (or rarely in introns or promoter region) can cause Crigler-Najjar disease type I or II. Approximately 60 mutations (point mutations, deletions, insertions) in the UGT1A1 gene have been identified,[146-150] indicating that Crigler-Najjar disease is genetically heterogeneous, while there is a homogeneity of its clinical presentation.

Phototherapy is the preferred long-term treatment for Crigler-Najjar disease type I, but has considerable disadvantages (see paragraph 7.1). If plasma UCB levels cannot be kept below 450-500 µmol/l, liver transplantation may be necessary to prevent irreversible brain damage due to kernicterus (see paragraph 7 for treatment options for Crigler-Najjar disease). During exacerbations of jaundice, several measures in addition to continuous high-intensity phototherapy are taken to manage the disease safely, including albumin infusion if the bilirubin-albumin molar ratio is above 0.7, and avoidance of drugs that displace bilirubin from albumin.[151] Before the introduction of phototherapy, all patients with Crigler-Najjar disease died from kernicterus.[140] In recent years, neurological outcome of Crigler-Najjar disease is good if treatment is started early and adequately. Combined data from recent surveys suggest that 23-47% of patients with Crigler-Najjar disease have neurologic damage ranging from mild to severe, 28-50% of patients will need one or multiple exchange transfusions, and 9-38% die of complications related to the disease.[151-155]

6. KERNICTERUS AND BIND

In 1847, Hervieux was the first to report yellow staining of brain nuclei in a severely jaundiced baby.[156] In 1875, Orth observed bilirubin pigment at autopsy in the brains of severely jaundiced infants.[157] The term *kernikterus* (from the German *kern*, nucleus, and the Greek *ikterus*, jaundice), was first used in 1903 by Schmorl, who described similar yellow staining of brain nuclei in infants who died with severe neonatal jaundice.[158] The regions commonly affected are the basal ganglia (globus pallidus, nucleus subthalamicus), the hippocampus, various nuclei in the brain stem (a.o. oculomotor, cochlear, vestibular and olivary nuclei) and cerebellum (nucleus dentatus).[159-161] Paragraph 3.1 discusses how bilirubin enters the brain and what determines bilirubin neurotoxicity.

Originally kernicterus was a pathologic diagnosis, later the term was also used for the acute and chronic neurological syndrome. Classic acute kernicterus in neonates is characterized by three phases.[16;81;162;163] In the first few days the infant becomes lethargic, hypotonic and sucks poorly. In the second phase, the infant becomes hypertonic with retrocollis and opisthotonus, frequently develops a fever and high-pitched cry, and may develop seizures. In the third phase, usually after one week, hypertonia gradually becomes less pronounced and is replaced by hypotonia. Chronic signs of kernicterus, so called long-term sequelae, include choreoathetosis, vertical gaze paralysis, sensorineural deafness and

dental dysplasia (the 'tetrad of Perlstein'),[90] asymmetric spasticity, motor delay and mental retardation. Subtle encephalopathy is referred to as bilirubin-induced neurologic dysfunction (BIND).[81;164] BIND can present with hearing loss, lowered IQ[165;166] and abnormal cognitive function.[167-170] Recently, plasma UCB levels up to ~510 µmol/l treated with phototherapy or exchange transfusion were not associated with adverse neurodevelopmental outcomes in infants born at or near term.[171]

Patients with Crigler-Najjar disease have a life-long risk of developing kernicterus. The risk increases especially during adolescence when phototherapy becomes less effective and compliance gets worse, and during intercurrent infectious illnesses. In some children with Crigler-Najjar disease type I there may be a late clinical presentation of bilirubin encephalopathy with cerebellar symptoms as presenting feature.[172]

7. TREATMENT OF UNCONJUGATED HYPERBILIRUBINEMIA / CRIGLER-NAJJAR DISEASE

7.1. Phototherapy

Phototherapy was discovered in 1956 when a nurse in England noticed that when jaundiced infants were exposed to sunlight they became less yellow.[173] Pediatric resident Cremer *et al.* subsequently demonstrated the efficacy of phototherapy by exposing preterm infants to blue fluorescent lights, which dropped plasma bilirubin levels.[173] In the mid 1960's other therapeutic trials followed[174] and since then phototherapy has been used extensively for treatment of unconjugated hyperbilirubinemia. The mechanism of phototherapy was studied in Gunn rats.[175-178] Phototherapy detoxifies bilirubin by converting UCB to photoisomers that are less hydrophobic than UCB. The photoisomers are a better substrate for Mrp2 and therefore can be excreted into bile without being conjugated first.[14;179] Phototherapy increases the amount of UCB in bile.[60;175;180]

When bilirubin molecules in the skin absorb (phototherapy)light, 3 photochemical reactions can occur: configurational and structural photoisomerization, (Figure 6) and photo-oxidation. In *configurational photoisomerization*, one (or both) of the double bonds at carbon atoms C4 and/or C15 in the bilirubin molecule is (are) opened, converting it from the ZZ configuration to a ZE, EZ or EE configuration (*Z* for *zusammen*, *E* for *entgegen*). When this occurs, the polar N and O groups are exposed, making the UCB-photoisomer less hydrophobic than UCB and therefore a better substrate for transport into the bile via Mrp2. The predominantly formed 4Z, 15E isomer is an unstable molecule that readily reverts back. This reverse reaction is relatively slow when the isomer is bound to albumin, but occurs rapidly in bile and intestinal lumen.[16] In s*tructural photoisomerization*, intramolecular cyclization of bilirubin occurs to form the non-reversible photoisomer lumirubin.[181;182] Lumirubin is cleared much more rapidly from plasma than the 4Z, 15E isomer, and is therefore considered mainly responsible for the decline in plasma UCB levels during

phototherapy.[183;184] *Photo-oxidation* of UCB involves hydroxylation and cleavage of –CH= bridges yielding mono- and dipyrroles that are small, polar and can be excreted in the urine.[185] Photo-oxidation is a slow process and appears to play a minor role in the photocatabolism of UCB *in vivo*.[16;185]

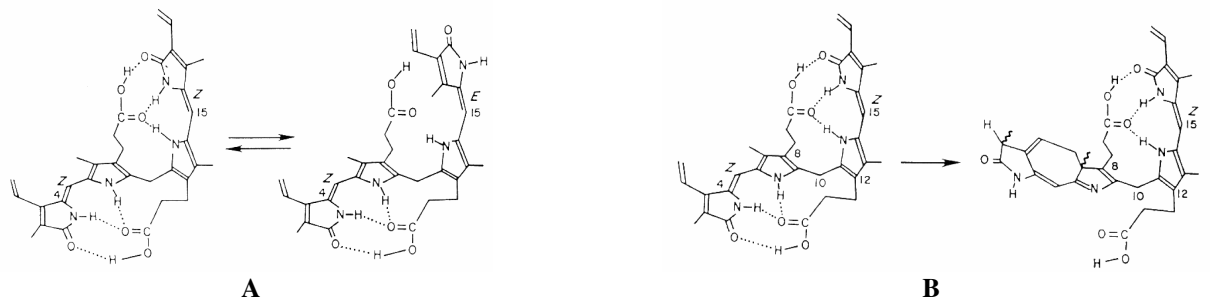


Figure 6. Phototherapy-induced photoisomerization of bilirubin. **A**: configurational photoisomerization. **B**: structural photoisomerization to lumirubin. From: McDonagh and Lightner.[14]

Several types of fluorescent lights have been used for phototherapy, including daylight, broad-spectrum, white, green, (special) blue and violet.[179;186] Efficacy results have been contradictory, but special (narrow spectrum) blue lights are generally considered superior because bilirubin absorbs light maximally in the blue range (from 420-500 nm) with a peak absorption at about 440-460 nm. White light is preferred by some clinicians because blue light distorts skin color, which makes it difficult to assess cynosis and jaundice in the neonate. Phototherapy is most efficient in the first 24-48 hours of treatment. The declining efficacy after 48 hours is probably related to configurational photoisomers that have been reverted to UCB, undergo enterohepatic circulation and increase the UCB load to be cleared by the liver.[16;187] Furthermore, phototherapy is less effective at lower plasma UCB concentrations due to depletion of the bilirubin pool in the skin, which is the main target for phototherapy.[188;189] The efficacy of phototherapy also depends on the body surface area exposed to the light. Therefore, double-sided phototherapy with a conventional overhead lamp plus a "biliblanket" reduces plasma UCB levels more rapidly.[16] Whether phototherapy should be given continuously or intermittently is not quite clear. Some studies reported continuous phototherapy to be more effective, but this was not confirmed by others. Since migration of bilirubin to the skin takes one to three hours[190] and is probably the rate-limiting step, intermittent phototherapy should be effective.[16] Intermittent phototherapy to *in vitro* human cells in tissue culture, however, caused more damage to DNA than continuous phototherapy.[191]

Since phototherapy was introduced almost 50 years ago, serious long-term side effects such as skin cancers have not been observed. However, phototherapy-induced DNA damage to human cell lines *in vitro* does occur and bilirubin was found to enhance this damage.[192;193] Short-term phototherapy has relatively minor side effects and is considered safe.[194]

Phenomena that have been attributed to or associated with phototherapy include retinal damage if the eyes are not shielded from light by eye patches,[195] diarrhea and decreased gut transit time,[196;197] increased insensible water loss,[198] temperature instability, patent ductus arteriosus[199;200] and the "bronze baby syndrome", which appears to be due to accumulation of photodegradation products (bilifuscins) when their biliary excretion is impaired by concomitant cholestasis.[119;201]

Patients with Crigler-Najjar disease type I have to undergo daily phototherapy up to 12 hours per day. Type II patients usually only need a few hours of phototherapy per day, if any. Long-term phototherapy has considerable disadvantages. Phototherapy becomes less effective with age, due to a decrease in surface area to body mass ratio,[151;202] due to a large tissue reservoir of UCB,[151] due to skin alterations,[145;155] and due to a diminishing compliance to the intensive phototherapy regimen which has a profound impact on the quality of (social) life.[155]

7.2. Exchange transfusion

Phototherapy has greatly reduced the need for exchange transfusion. With this technique, approximately 85% of circulating red blood cells will be replaced (when replacing 160 ml/kg BW), and plasma UCB levels will generally be reduced by 50%.[119] The exchange transfusion physically removes defective red bloods cell and UCB, which diffuses from the extravascular space (*i.e.* tissue pool) into plasma. Indications for exchange transfusion include symptoms and signs characteristic of acute bilirubin encephalopathy (kernicterus; see paragraph 5), dangerously high or rapidly rising plasma UCB concentrations despite phototherapy, and progressive anemia due to hemolysis.[203;204] The mortality rate from the procedure is around 0.3%. Significant morbidity is associated with ~5% of exchange transfusions.[205;206] Complications include cardiac and vascular complications such as cardiac arrest and thrombosis of the portal vein in case the exchange transfusion was done via a catheter in the umbilical vein, metabolic and coagulation disturbances, transmission of infectious diseases, graft versus host disease and necrotizing enterocolitis.

In the management of Crigler-Najjar disease, generally exchange transfusions are not required. Sometimes exchange transfusions are used in the neonatal period when the diagnosis is not yet clear. Incidentally, exchange transfusions are performed when plasma UCB concentration is dangerously increased and/or albumin concentration decreased, for example during intercurrent (febrile) illnesses or around surgery.[151;155]

7.3. Phenobarbital

Phenobarbital is an anti-epileptic drug that enhances the three steps in hepatic bilirubin metabolism independently: uptake and storage of UCB by the hepatocyte, conjugation, and biliary secretion.[30;176;207-209] Net uptake and storage is enhanced via an increased concentration of ligandin. Conjugation is enhanced via induction of UGT1A1. Biliary secretion is most likely enhanced due to induction of Mrp2. Phenobarbital is a CAR (constitutive androstane

receptor) agonist. Wagner et al.[210] showed that phenobarbital, and other CAR agonists, induce Mrp2.

Phenobarbital is used to distinguish between type I and II Crigler-Najjar disease. In type I patients, phenobarbital is not effective because there is no residual enzyme activity that can be enhanced. In the animal model of type I Crigler-Najjar disease, phenobarbital decreases plasma UCB levels, despite the absence of residual enzyme activity, but this has been demonstrated to be due to a shift of the bilirubin pool to the liver.[144] Phenobarbital is effective in type II Crigler-Najjar disease. It usually decreases plasma UCB concentration by 30% or more.[142] Side effects include sedation, and induction of cytochrome P450 enzymes which accelerate the metabolism of many drugs, vitamins, clotting factors and estrogenic and androgenic hormones.[176]

Apart from its use for treatment of type II Crigler-Najjar disease, phenobarbital is not used anymore for treatment of unconjugated hyperbilirubinemia. Originally, it was given to pregnant mothers before delivery or to the infant within 24 hours after birth to limit the severity of unconjugated hyperbilirubinemia and the need for exchange transfusions.[211-214] However, phototherapy is more effective than phenobarbital and combining phototherapy with phenobarbital did not reduce plasma UCB levels more rapidly than phototherapy alone.[215] Furthermore, the effect of phenobarbital does not start until a few days after administration.[211]

7.4. Decreasing UCB production

UCB production can be decreased via inhibition of heme oxygenase (HO), the rate-limiting enzyme in the catabolism of heme to UCB. In theory, inhibition of biliverdin reductase could also be used to decrease UCB production. However, inhibitors of biliverdin reductase have not been explored, probably because their use would cause green babies. HO inhibitors such as tin (Sn)- and zinc (Zn)-protoporphyrin and -mesoporphyrin are synthetic heme analogues.[216] A single dose inhibits HO for several days. The inhibition of heme degradation does not result in accumulation of heme because heme is excreted into bile.[217] Sn-mesoporphyrin is the preferred HO inhibitor for treatment of neonatal jaundice,[218-221] but is currently not recommended for routine treatment because of insufficient evidence,[222] and unknown long-term safety.[223] Recent trials focused on treatment of mice.[224;225] Results of new trials in neonates are awaited. In neonates with glucose-6-phosphate dehydrogenase deficiency (G6PD), Sn-mesoporphyrin supplants the need for phototherapy to control hyperbilirubinemia.[226] Side effects of Sn-protoporphyrin include photosensitization, which can accelerate the destruction of UCB by light but can also cause cutaneous erythema.[219;227] Phototoxicity involves production of free radicals and other reactive oxygen species which can cause cell damage, presumably via accelerated lipid peroxidation.[228]

Sn-mesoporphyrin and Sn-protoporphyrin have been used in the management of Crigler-Najjar disease,[229;230] but results were temporary and disappointing. In Crigler-Najjar patients,

early administration of heme oxygenase inhibitors is expected to be more effective than initiation in adolescence, because in the latter case, total-body amount of UCB is many times greater than the amount of UCB in the intravascular space.[16]

7.5. Intestinal capture of UCB

UCB gets into the intestinal lumen via one of three routes: 1) biliary secretion of conjugated bilirubin, subsequently deconjugated to UCB; 2) biliary secretion of UCB: a very small amount of UCB can be excreted into bile (see paragraph 2.6);[11;34] 3) transepithelial diffusion: UCB can diffuse from the blood into the intestinal lumen across the intestinal mucosa along concentration gradients, particularly when plasma UCB concentrations are high as in Crigler-Najjar disease (see Figure 1 in Chapter 2).

Intestinal capture of UCB followed by fecal excretion reduces the enterohepatic circulation of UCB and subsequently decreases plasma UCB concentration. Several orally administered non-absorbable binders of UCB have been applied for intestinal capture. Agar,[231] activated charcoal[232] and cholestyramine[233] are no longer used for treatment of unconjugated hyperbilirubinemia because of inconsistent clinical results and side effects.[234-236] Zinc sulphate was shown to decrease plasma UCB levels in patients with Gilbert syndrome, but serum zinc levels increased simultaneously.[237] Zinc methacrylate did not increase serum zinc levels, but was less effective in Gunn rats than zinc sulphate.[238] Intestinal capture of UCB by calcium phosphate was very effective in Gunn rats,[239] but efficacy was less pronounced in patients with Crigler-Najjar disease.[145]

We hypothesized that fat could be used to capture UCB in the intestine, considering the relatively lipophilic character of UCB.[139] In this thesis we investigated whether stimulation of fecal fat excretion by orlistat (see paragraph 9) decreases plasma UCB concentrations in Gunn rats and patients with Crigler-Najjar disease (see chapter 2).

7.6. Bilirubin oxidase

Bilirubin oxidase was used in several experimental ways for treatment of unconjugated hyperbilirubinemia. UCB was removed from rat- or human blood by passage through a filter containing bilirubin oxidase,[240] bilirubin oxidase was fed to Gunn rats,[241] and PEG-bilirubin oxidase was injected i.v. in Gunn rats.[242] As mentioned in paragraph 2.8, induction of cytochrome P450-1a1 (Cyp1a1) by TCDD decreases plasma UCB concentration in Gunn rats. Treatment of Gunn rats with indole-3-carbinol also induces the oxidative pathway of UCB metabolism.[243] Retention of UCB itself induces this pathway as well.[62] Several naturally occurring indoles extracted from cruciferous vegetables, such as cabbage, cauliflower and sprouts induce P4501a1 and 1a2 in rat liver and intestine.[16;244] Bilirubin oxidase administration or induction of bilirubin oxidation is currently not applied as therapeutic strategy for neonatal jaundice or Crigler-Najjar disease.

7.7. Hepatocyte transplantation

Since liver architecture and function, except for deficiency of UGT1A1 activity, are normal in Crigler-Najjar disease type I, hepatocyte transplantation might be safer and less invasive than liver transplantation. Correction of Crigler-Najjar disease requires only partial replacement of UGT1A1 activity.[245] In Gunn rats, several techniques have been investigated, such as infusion of (unaffected) hepatocytes into the portal vein, or via intraperitoneal injection.[246-249] Hepatocyte transplantation temporarily decreased plasma UCB concentration in Gunn rats.[247] So far, hepatocyte transplantation has been performed in two patients with Crigler-Najjar disease type I. The first patient was a 10 year old girl in whom UGT1A1 activity was restored to 5.5% of normal after hepatocyte transplantation via percutaneous infusion through the portal vein. Afterwards, maximum plasma UCB levels dropped from 455 to 239 µmol/l, and she required 6-7 hours of phototherapy instead of 10-12 hours.[250] Long-term results are awaited. More recently, the second patient, a 9 year old boy, received an allogenic hepatocyte transplantation.[251] Initially, plasma UCB levels decreased from 530 to 359 µmol/l. However, he was treated for cellular rejection and later he received a liver transplantation because of poor compliance to phototherapy. Although hepatocyte transplantation was safe and partially effective in these two patients, problems with long-term efficacy, rejection and immune suppression may prevent future use in Crigler-Najjar disease.

7.8. Liver transplantation

Several patients with Crigler-Najjar syndrome type I have undergone liver transplantation.[252-257] Successful liver transplantation effectively restores UGT1A1 activity which results in low or normal plasma UCB levels and eliminates the need for phototherapy. However, these benefits have to be weighed against the risks and complications of liver transplantation. The one year survival after liver transplantation is between 85 and 90%,[258] although over the past years survival has improved.[259;260] Possible complications include rejection, infection, bleeding, thrombosis and biliary complications.[258] To reduce the risk of rejection, patients receive life-long immunosuppressive medication, which increases the risk of lymfoproliferative disease and late infections, and has side effects as nephrotoxicity and hyperlipidemia.[260] Two types of liver transplantation are used. In orthotopic liver transplantation the patient's own liver is removed and a donor liver is inserted in its place. In auxiliary liver transplantation, (part of) the patient's own liver is left in situ, but supported by the transplantation of a non-affected donor graft.[261;262] The theoretical advantage of the latter procedure is that, if gene therapy would become available in the future, this could still be applied to the native liver, allowing possible withdrawal of immunosuppression.

7.9. Gene therapy

Since Crigler-Najjar disease is caused by molecular lesions of a single gene and partial enzyme replacement would be enough to significantly lower plasma UCB concentrations,

gene therapy would be an elegant potential therapeutic option. However, vector toxicity and concerns about long-term safety have so far prevented the use of gene therapy in patients.

The structure of the UGT1A1 gene has been elucidated and the gene was successfully cloned in 1991.[263-265] Since then many gene transfer strategies have been evaluated in Gunn rats. Early generation adenoviral vectors effectively corrected UGT1A1 activity,[266;267] but the effects were transient. Several strategies to prolong the duration of transgene expression have been explored, including induction of tolerance[268] and expression of immunomodulatory molecules.[269] However, acute toxicity and immunogenicity of viral proteins were a major disadvantage of these vectors. Subsequently, helper-dependent, or "gutless" adenovectors were developed that have negligible chronic hepatic toxicity.[270] A single i.v. injection successfully corrected unconjugated hyperbilirubinemia in Gunn rats for more than 2 years. One of the side effects was transient thrombocytopenia. Other viruses that have been used as vectors include retrovirus,[271;272] lentivirus[273;274] and recombinant simian virus.[275] Non-viral strategies such as chimeraplasty,[276] liposomes,[277] and plasmids[278-280] have been evaluated. *Ex-vivo* gene therapy with transplantation of manipulated fibroblasts corrected the gene defect but resulted in animals developing tumors.[281] Before any of the above strategies can be used for gene therapy in patients with Crigler-Najjar disease, long-term safety will need to be confirmed.

8. GUNN RAT

In this thesis we used the Gunn rat for our animal studies. In 1938, Gunn first described a spontaneously mutant rat strain with recessively inherited hyperbilirubinemia within a colony of Wistar rats.[282;283] A colony of these rats was maintained for over 15 years. It was not until 1957, when defective bilirubin glucuronidation was reported, that the Gunn rat was recognized as an animal model of Crigler-Najjar disease type I[284;285] (Figure 7).

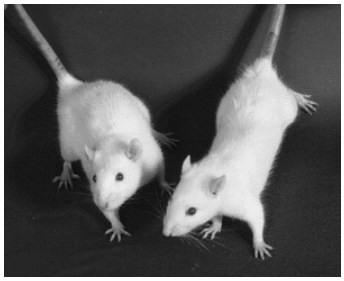


Figure 7. Gunn rats.

The Gunn rat is now a well-established and frequently used model of inherited unconjugated hyperbilirubinemia. Advantages of the model include the ability to use heterozygous littermates (Jj) of affected homozygous Gunn rats (jj) as matched controls.[69] Different strains of Gunn rats exist.[286;287] In our experiments we used albino Gunn rats (RHA/jj).

Although all Gunn rats have degenerative lesions of the brain, not all develop gross disturbances of gait or other signs of kernicterus.[288] The neuropathological lesions in Gunn rat pups are similar to those in humans, with cell loss and gliosis in auditory and oculomotor nuclei, cerebellum, hippocampus and basal ganglia.[69] As in humans, brainstem auditory evoked potentials (BAEPs) are a sensitive indicator of bilirubin neurotoxicity in Gunn rats.[289] In contrast to human kernicterus, cerebellar hypoplasia is a prominent feature of UCB damage in Gunn rats, and the cause of ataxia.[290;291] Besides brain damage, high UCB concentrations can cause renal papillary necrosis. High UCB levels in the medulla interfere with sodium and water transport, resulting in impaired urinary concentration with polyuria.[87;88]

Figure 8. Gunn rats receiving phototherapy in one of our experiments. Blue phototherapy lamps were suspended in a reflective canopy 20 cm above the bottom of the cage. The Gunn rats were shaven on their backs and flanks.

9. ORLISTAT

This paragraph provides background information on orlistat which was investigated in this thesis as option for oral treatment of unconjugated hyperbilirubinemia. We used orlistat to increase fecal fat excretion, hypothesizing that fat could be used to capture UCB in the intestinal lumen (see paragraph 7.5 and chapter 2).

Orlistat (Xenical®; Figure 9) is a selective inhibitor of gastrointestinal lipases that dose-dependently inhibits hydrolysis of dietary triglycerides.[292] It is a chemically synthesized derivative of the natural product lipstatin and specifically inhibits lipases at their catalytic triad by covalent binding to the serine residue.[293] Orlistat has little or no activity against amylase, trypsin and phospholipases.[292] At the recommended dose of 3 times daily 120 mg for adults, dietary fat absorption is reduced by approximately 30%. Orlistat acts locally in the gastrointestinal tract and systemic absorption is minimal (~1%).[294] Orlistat is applied for treatment of obesity and obesity-related co-morbid conditions. In combination with dietary intervention and exercise, orlistat is used for management of weight loss and weight maintenance. Orlistat treatment is associated with beneficial effects on cardiovascular risk

factors including dyslipidemia, decreased insulin sensitivity and hypertension.[294-296] Numerous clinical trials in adults have not reported serious side effects.[294] Rather, side effects are generally mild to moderate, temporary and limited to gastrointestinal effects such as fatty/oily stool, flatulence, and abdominal pain. Recently, orlistat has been introduced in the European Union for treatment of obese adolescents.[297] Clinical trials in obese adolescents[298-301] and prepubertal children[302] indicate that orlistat treatment is well-tolerated by children and has a side effect profile similar to that observed in adults. Orlistat had no significant effect on the balance of six selected minerals in obese adolescents.[303] Besides being an inhibitor of gastric and pancreatic lipases, orlistat was recently reported to be an inhibitor of fatty acid synthase, thereby halting tumor cell progression.[304]

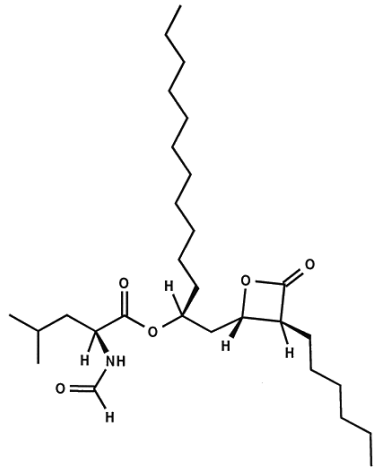


Figure 9. Structure of orlistat.

10. BILE SALTS

This paragraph provides background information on bile salts which were investigated in this thesis as option for oral treatment of unconjugated hyperbilirubinemia.

Bile salts are quantitatively the major organic constituents of bile. Bile formation is crucial for hepatobiliary secretion of bilirubin. The liver synthesizes bile salts from cholesterol. In addition to their role in enhancing bile flow and the biliary secretion of exogenous and endogenous organic compounds, including phospholipids and cholesterol, bile salts are important for intestinal absorption of dietary fats and fat-soluble vitamins (A, D, E, K).[305] Bile salts are secreted into bile by the canalicular bile salt export pump (Bsep, Abcb11).[306] In the intestine, more than 95% of bile salts are efficiently reabsorbed into the enterohepatic circulation.[307] Intestinal absorption is principally mediated by the apical sodium-dependent bile salt transporter (Asbt, Slc10a2) in the terminal ileum.[308] Uptake of bile salts by the liver is mainly mediated by the Na^+ taurocholate cotransporting polypeptide (Ntcp, Slc10a1).[309]

In humans and rats, the major newly synthesized (primary) bile salts are cholic acid (CA) and chenodeoxycholic acid (CDCA) After synthesis, more than 99% of primary bile salts are conjugated with either the amino acid taurine or glycine, which increases hydrophilicity. In the intestine, conjugated CA and CDCA can undergo deconjugation and subsequent dehydroxylation by the bacterial flora, resulting in the toxic secondary bile salts deoxycholate and lithocholate, and in the tertiary bile salt ursodeoxycholic acid (UDCA). UDCA is a hydrophilic, non-toxic bile salt compared with the hydrophobic, toxic CA. UDCA inhibits UCB-induced apoptosis in cultured rat neural cells.[310]

UDCA is used in the management of cholestatic liver diseases with conjugated hyperbilirubinemia. We propose that UDCA might be used for treatment of unconjugated hyperbilirubinemia as well. Solubilization of UCB by bile salts and interactions between UCB and bile salts occur.[311-313] Furthermore, dietary supplementation with UDCA has been suggested to impair fat absorption in some individuals.[314] Since we hypothesized that increasing fecal fat excretion reduces the enterohepatic circulation of UCB via intestinal capture of UCB by fat, we investigated whether UDCA treatment decreases plasma UCB concentrations in Gunn rats (see chapter 2). In contrast to our hypothesis, Méndez-Sánchez *et al.* have hypothesized that dietary UDCA supplementation induces enterohepatic cycling of UCB by causing bile salt malabsorption, which elevates colonic bile salt levels, promoting solubilization and reabsorption of UCB.[315] Their hypothesis has not been proven by ^{3}H-UCB kinetic studies.

This chapter mainly discussed bilirubin metabolism and treatment options for unconjugated hyperbilirubinemia in Crigler-Najjar disease to provide background information regarding the research described in this thesis. We propose two oral treatment options for unconjugated hyperbilirubinemia: orlistat, and bile salts. In chapter 2 the outline of this thesis is presented.

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